



113
173
THS



This is to certify that the

thesis entitled

GALACTOSE OXIDATION
IN RAT LIVER MICROSOMES

presented by

Nancy Ann Josef

has been accepted towards fulfillment
of the requirements for

M.S. degree in Biochemistry

A handwritten signature in cursive script, appearing to read "W. W. Wells", written over a horizontal line.

Major professor

Date Mar. 28/78

GALACTOSE OXIDATION
IN RAT LIVER MICROSOMES

By

Nancy Ann Josef

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1978

9113417

ABSTRACT

GALACTOSE OXIDATION IN RAT LIVER MICROSOMES

By

Nancy Ann Josef

Galactose oxidation was investigated in rat liver models to account for the production of galactonic acid in patients exhibiting classical galactosemia and in patients receiving galactose tolerance tests.

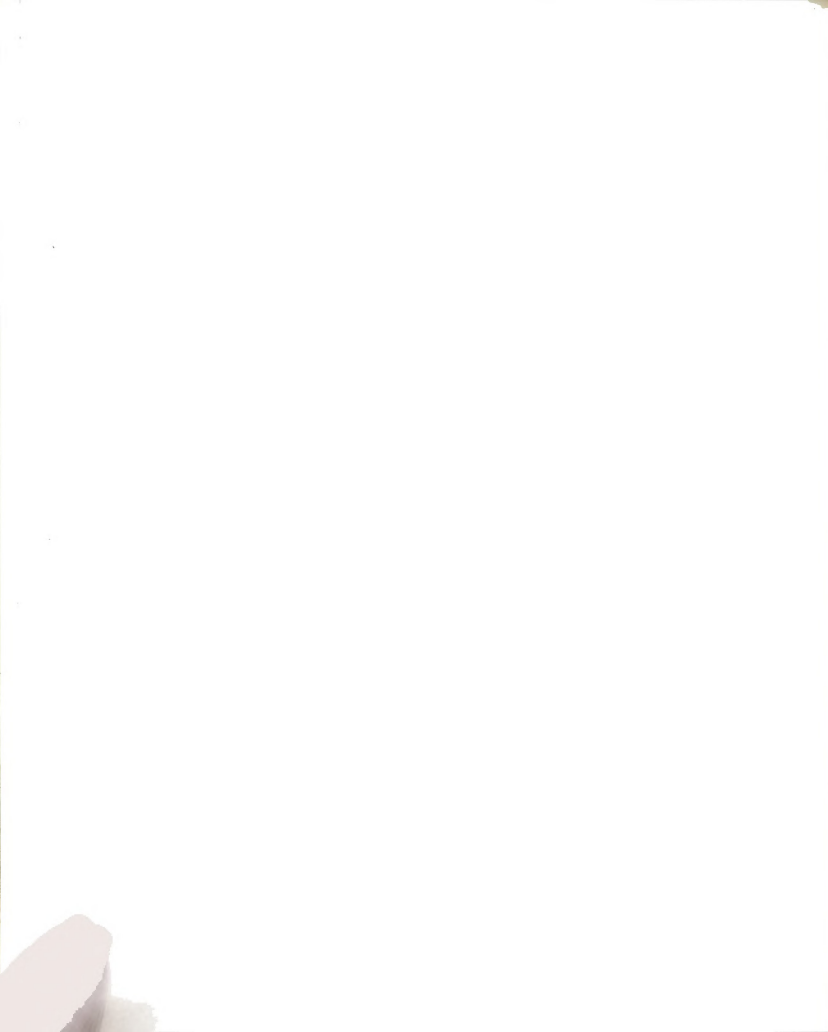
A gas chromatographic assay was devised for the identification and quantitation of galactonic acid. Rats maintained on a 40% galactose diet excreted more than 30 mg of galactonate per day in the urine and accumulated galactonic acid in several tissues. Liver microsomes incubated with 30 mM galactose produced galactonic acid. This oxidizing activity was also seen in the mitochondrial fraction; the soluble fraction had no activity. Optimal activity occurred at pH 8.0, and inhibition was caused by heavy metals and sulfhydryl reagents. The apparent K_m for galactose was 32.9 mM and V_{max} was 160 nmoles of galactonic acid/4 hr/mg protein. The activity was specific for galactose although unidentified oxidation products of altrose, talose, and 2-deoxy-galactose were detected. Oxygen appeared to be the sole hydrogen acceptor; galactose dependent formation of hydrogen peroxide was demonstrated during the incubation period. These data suggest that an enzyme with galactose oxidase activity is present in rat liver microsomes.

ACKNOWLEDGEMENTS

I wish to express sincere appreciation to Dr. William W. Wells for his enthusiasm, patience, guidance, and financial support throughout the course of these experiments. I thank my committee members Dr. Loran L. Bieber and Dr. Richard L. Anderson for taking the time to help in this endeavor. I wish to thank Thomas for his love and encouragement, and for having more confidence in me than I have in myself.

TABLE OF CONTENTS

	Page
List of Tables.	v
List of Figures	vi
List of Abbreviations	vii
INTRODUCTION	
Rationale and Objectives	1
Literature Survey.	2
MATERIALS AND METHODS	
Materials	7
Hemoglobin Incubations	8
Cell Incubations	8
Animal Maintenance	9
Tissue Collection.	11
Microsome Preparation	11
Conditions of Incubations	12
Difference Spectra	12
Galactonate Isolation	12
Galactonate Determinations.	13
Hydrogen Peroxide Assay.	14
Protein Separation	14
RESULTS	
Hemoglobin and Blood Studies	16
<u>In Vivo</u> Studies	16
Microsome Activities.	18
Cellular Distribution	18
Galactonate Production with Time, Protein and Substrate	20
Effect of pH	20



Activators and Inhibitors	20
Substrate Specificity.	26
Difference Spectra.	26
Reaction with Hydrogen Acceptors	26
Formation of Hydrogen Peroxide.	28
Effect of Age	29
Distribution in Species	29
Protein Separation.	29
DISCUSSION.	33
REFERENCES.	41



LIST OF TABLES

Table		Page
I.	Diet Composition.	10
II.	Concentrations of Galactonate Found in Tissues of Rats Fed High Galactose Diets and Phenobarbital Water	17
III.	Galactose Oxidation Activity in Rat Liver Microsomes.	19
IV.	Effects of Various Activators and Inhi- bitors on the Galactose Oxidizing Activ- ity of Rat Liver Microsomes	24
V.	Effects of Hydrogen Acceptors on the Galactose Oxidizing System in Rat Liver Microsomes.	27
VI.	Effects of Age on Galactose Oxidation in Rat Liver Microsomes	30
VII.	Galactose Oxidation in Various Species	31
VIII.	Purification of the Hydrogen Peroxide Producing Activity from Rat Liver Microsomes.	32



LIST OF FIGURES

Figure	Page
1. Time Course of Microsomal Galactonate Production.	21
2. Lineweaver-Burk Analysis of Kinetic Data	22
3. Effect of pH on Microsomal Galactose Oxidation	23

LIST OF ABBREVIATIONS

BHT	butylated hydroxytoluene
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
K_m	Michaelis constant
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinu- cleotide phosphate
SDS	sodium dodecyl sulfate
TMS	trimethylsilyl
Tris	tris (hydroxymethyl) aminomethane
V_{max}	maximal velocity



INTRODUCTION

Rationale and Objectives

The excretion of galactonic acid by patients with classical galactosemia and by subjects given galactose tolerance tests has been reported (1). The mechanism for this oxidation and the conversion of galactose to carbon dioxide in galactosemics has been widely disputed (2-7).

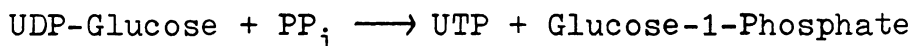
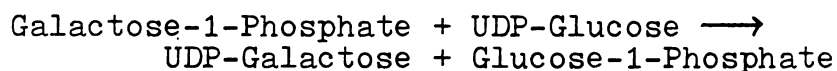
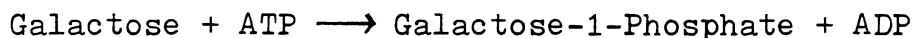
Work in this laboratory has shown that high levels of galactose lead to impaired phagocytosis and oxygen-dependent killing of bacteria in polymorphonuclear leukocytes (8,9). In the course of those studies it was found that an in vitro Fenton reaction combining hydrogen peroxide and ferrous iron produced hydroxyl radicals that could convert galactose to galactonic acid (10). Since it is known that liver microsomes are capable of producing hydrogen peroxide and hydroxyl radicals (11), the liver microsome system was studied to determine if a similar hydroxyl radical mechanism could produce galactonic acid.

To test this hypothesis, experiments were conducted using microsomes isolated from rat liver. Levels of galactose employed in these studies were comparable to those encountered in galactosemics (12,13). When it was shown that an in vitro microsomal system could indeed produce galactonic acid,

various activators and inhibitors were used in attempts to elucidate the mechanism. The in vitro results of the microsomal incubations were extrapolated to the in vivo system by dietary studies. The observed hydrogen peroxide production along with galactonic acid production led to the proposal that a protein with galactose oxidase activity was responsible for the microsomal oxidation of galactose.

Literature Survey

Galactose is normally metabolized in most biological systems by conversion to glucose through the Leloir pathway (14-17):



Galactose reacts with ATP to form galactose-1-phosphate and ADP. The equilibrium is far in the direction of sugar phosphorylation but the reaction is reversible (18). In the second step of the galactose-glucose interconversion, the enzyme galactose-1-phosphate uridylyltransferase reacts with uridine diphosphate glucose (UDP-glucose) and galactose-1-phosphate to give UDP-galactose and glucose-1-phosphate. The conversion of galactose to glucose by inversion of the hydroxyl group at the fourth carbon of the hexose chain is catalyzed by uridine diphosphate galactose-4-epimerase. The last step

in the pathway is catalyzed by uridine diphosphate glucose pyrophosphorylase; UDP-glucose and pyrophosphate react to form glucose-1-phosphate and UTP. This reaction is reversible, allowing not only the original carbon chain of galactose to enter the pathway of glucose metabolism, but also enables UDP-glucose to be formed from glucose and UTP (19).

Classical galactosemia is an inherited disease resulting from a deficiency in galactose-1-phosphate uridylyltransferase. The first detailed description of the syndrome by Mason and Turner in 1935 (20) was followed by numerous reports clearly establishing the clinical entity (21-25).

The most common initial clinical symptom is failure to thrive, and occurs in almost all cases. Vomiting or diarrhea usually starts within a few days of milk ingestion (23). Jaundice and hepatomegaly present after the first week of life and are accentuated by the severe hemolysis seen in some patients. Cataracts are observed a few days after birth, and retarded mental development is apparent in those first observed after the first several months of life (19). Chemical findings include elevated blood galactose, galactosuria, hyperchloremic acidosis, and amino aciduria (26,27). The acidosis may be secondary to the gastrointestinal disturbances, but may be a result of renal tubular dysfunction and a defect in urine acidification mechanisms (26). Albuminuria (27) and amino aciduria (28,29) are the result of a renal toxicity syndrome. Classical galactosemics who are never exposed to galactose should exhibit no abnormalities. The clinical symptoms are most likely the result of the accumulation of

galactose-1-phosphate and the products of alternate pathways of galactose metabolism (19,30), but in many instances the reason for the toxicity of an organ, especially the liver and brain, remain obscure (19).

One alternate route of galactose metabolism involves the reduction of galactose to galactitol. This reaction requires NADPH and is catalyzed by aldose reductase. The K_m value for galactose is between 12 and 20 mM (31,32), a level that is surpassed in galactosemia (12,13). Galactitol has been isolated from the tissues and urine of galactosemics (33-35) and administration of radioactive galactitol to normal patients has shown that the compound is not further metabolized or converted to carbon dioxide, but only excreted in the urine (36). The formation of galactitol in the lens has been directly linked to cataract formation (37,38). Since galactitol diffuses very slowly from the lens, it is osmotically active and there is an obligatory movement of water into the lens fiber, contributing to the cataract formation (39).

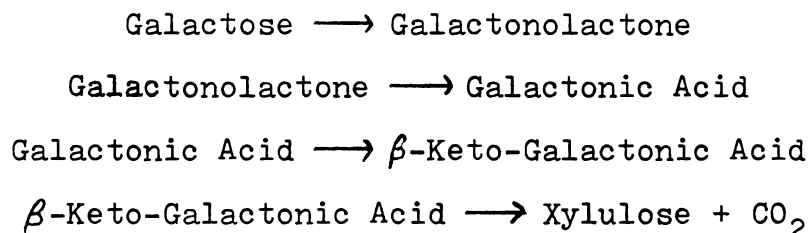
Some patients who appear to have a total absence of galactose-1-phosphate uridylyltransferase are still able to oxidize small amounts of labelled galactose to carbon dioxide (40,41). It has been shown that galactosemic fibroblasts produce $^{14}\text{CO}_2$ from galactose-1- ^{14}C (42), and radioautography of cultured human galactosemic and normal cells also suggests the possibility of an alternate pathway for galactose metabolism (43).

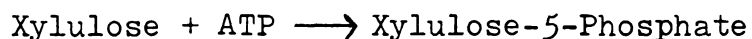
An auxiliary pathway for galactose metabolism was pro-

posed by Isselbacher (44,45) where galactose-1-phosphate and uridine triphosphate react to form UDP- galactose and pyrophosphate. This reaction is catalyzed by uridine diphosphate galactose pyrophosphorylase. The action of this enzyme could circumvent the block in transferase deficiency, but it was later shown that the activity of this enzyme in normal human liver is low (46), and the activity in liver biopsy specimens from galactosemics is insignificant (47).

Inouye and coworkers reported the presence of galactose-6-phosphate in galactosemic erythrocytes (48), but this has been disputed (49). Galactose-6-phosphate has been shown to be formed from galactose-1-phosphate by phosphoglucomutase (50,51), or by hexokinase (52) in in vitro systems, but the rate of formation is very small. The enzyme hexose-6-phosphate dehydrogenase oxidizes galactose-6-phosphate to 6-phosphogalactonic acid (53); the latter was shown to be a dead end product of metabolism (5). Another liver enzyme, galactose-6-phosphate dehydrogenase oxidizes galactose-6-phosphate to a ketoaldose product (54). There is no direct evidence to suggest that this enzyme is involved in any in vivo pathway of galactose metabolism.

Another pathway explaining galactose metabolism in galactosemics was proposed by Cuatrecasas and Segal (2):





Galactose is converted to galactonolactone by an NAD^+ requiring enzyme, galactose dehydrogenase. A lactonase acts upon the lactone to produce galactonic acid, which proceeds through an unstable intermediate, β -keto-galactonic acid to xylulose and carbon dioxide. The enzyme β -L-hydroxy acid dehydrogenase was proposed to catalyze this step (2). Xylulose can be readily phosphorylated by liver xylulokinase to xylulose-5-phosphate.

Galactose dehydrogenase was isolated and partially purified from rat liver cytosolic fraction (3) and characterized as an enzyme inhibited by divalent metal ions, sulhydryl reagents, and exhibited a broad substrate specificity (4). However, other researchers have shown that the galactose dehydrogenase was actually alcohol dehydrogenase, and that the increase in absorbance at 340 nm used to assay the activity was a result of alcohol contamination of sugar substrates (5-7). When alcohol was removed from the sugars, no activity was observed. In addition the isolation of galactonic acid from an elaborate reaction incubation involving hydrogen peroxide, peroxidase, and resorcinol, along with galactose, purified enzyme, and NAD^+ , could not be reproduced if only galactose, enzyme, and NAD^+ were employed (5).

Because of such conflicting data as outlined above, the mechanism of the oxidation of galactose to galactonic acid and carbon dioxide in totally transferase deficient galactosemics remains to be elucidated.

MATERIALS AND METHODS

Materials. All reagents used were analytical grade. Horse-radish peroxidase (EC 1.11.1.7) and catalase (EC 1.11.1.6) were from Worthington Biochemical Co. Catalase was purified before use by passing through Sephadex G-10. Superoxide dismutase (EC 1.15.1.1) was obtained from Sigma Chemical Co. Bovine hemoglobin was isolated earlier in this laboratory and stored at -20 degrees. D-Galactose, D-mannose, D-glucose, D-xylose, D-arabinose, D-fructose, nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate (NADP^+), reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), D-ribonic acid- δ -lactone, 2,6-dichlorophenolindolphenol, o-dianisidine dihydrochloride, pyrazole, and butylated hydroxytoluene (BHT) were from Sigma. Galactose was found to be free of galactonic acid contamination by gas chromatography. Galactono- δ -lactone was from General Biochemicals, hydrogen peroxide from Mallinckrodt, diphenylfuran from Eastman, and diphenylisobenzofuran from Aldrich. D-Talose, D-allose, D-altrose, and L-galactose were gifts from Dr. W. A. Wood. All rats were obtained from Holtzman (Madison, WI), and adult male guinea pigs were from Elm Hill Laboratories (Cambridge, MA). One week old chicks were

were the gift of Dr. D. Polin. Mice of the C57BL/6J-+/- strain were purchased from Jackson Laboratories (Bar Harbor, ME). Beef liver was obtained at a local slaughter house.

Hemoglobin Incubations. Bovine hemoglobin was dissolved in 250 mM potassium phosphate, pH 7.4, to a concentration of 50 mg/ml and centrifuged to remove insoluble impurities. Sodium dithionite was added to reduce iron, and the solution was passed over Sephadex G-25. Hydrogen peroxide solutions were made fresh and the concentration was determined spectrophotometrically ($\epsilon_{230\text{ nm}} = 81\text{ M}^{-1}$). Reduced hemoglobin (1.2 mg/ml), 2.0 mM hydrogen peroxide, and 30 mM galactose or glucose were incubated in 250 mM potassium phosphate, pH 7.4, in a total volume of 1.0 ml, for 2 hr at 37 degrees. Protein was precipitated with 200 μ l of 30% trichloroacetic acid (TCA). Samples were centrifuged and the supernate was extracted three times with three volumes of diethyl ether. A few drops of 0.1 M NaOH were added to convert the products to the sugar anionic forms. The samples were dried under nitrogen and trimethylsilyl (TMS) derivatives were prepared as previously described (55). Gas chromatography was performed on the aldonate derivatives at 180 degrees using a Hewlett Packard model 5830 A gas chromatograph equipped with a 1.8 m x 2 mm glass column packed with 3% OV-1 on Gas Chrom Q, 80-100 mesh (Applied Science Laboratories, Inc., State College, PA).

Cell Incubations. Rats and guinea pigs were ether anesthe-

tized and blood collected by heart puncture was placed in heparinized tubes. Blood was centrifuged at 5000 x g, plasma was removed, and red cells were resuspended to the original blood volume in Krebs-Ringer phosphate solution (without calcium or magnesium to prevent clumping), pH 7.4 (56). Red cells (3.0×10^9) or plasma (0.5 ml) were incubated with 1.0 mM hydrogen peroxide and 30 mM galactose in a total volume of 1.0 ml for 4 hr at 37 degrees. Samples were protein precipitated with 200 ul 30% TCA, and samples were treated as outlined above.

Animal Maintenance. The effects of phenobarbital on microsomal galactose oxidizing activity and the tissue distribution of galactonate were observed through diet studies. Male rats weighing 250-300 g were randomly divided into two groups, fed a commercial chow diet (Allied Mills, Inc., Chicago, IL), and were allowed to drink distilled water or water containing 0.1% phenobarbital, pH 7.0, ad libitum. After 7 days these groups were randomly subdivided, and the rats were placed in individual stainless steel metabolism cages. One half of each group was maintained on a control or 40% galactose containing diet (Table I) for an additional 72 hours, and the water regimen was continued. The diets were fed ad libitum. A supplemental salt mixture was added to bring the essential elements up to accepted levels. Urine was collected under toluene for the last 24 hours before sacrifice.

TABLE I
Diet Composition^a

Component	Concentration (g/kg)
Galactose or Sucrose	400
Vitamin-Free Casein	250
Sucrose	226
Soybean Oil	50.0
Phillips-Hart Salt Mixture ^b	50.0
Alpha Cellulose	12.5
Vitamin Mix	10.0
Choline Chloride	1.0
Supplemental Salt ^c	0.5

^aNo galactonate contamination was found in the diets.

^bThe Phillips-Hart salt mixture (Teklad Test Diets) consisted of (% by weight): 30.00% CaCO_3 , 7.50% $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, 0.005% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.003% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 32.2% K_2HPO_4 , 2.75% ferric citrate, 10.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08% KI, 16.7% NaCl, and 0.025% ZnCl_2 .

^cThe supplemental salt mixture consisted of (% by weight): 6.123% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.500% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 14.004% ZnO, 38.348% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, and 41.035% alpha cellulose.

Tissue Collection. Animals were anesthetized with diethyl ether and blood taken by heart puncture was placed in heparinized tubes. Livers were removed and samples (2 g) were minced in 10-15 ml ice cold 0.15 M potassium phosphate buffer, pH 7.4, for microsomal preparation. Intestines were removed, rinsed with distilled water, and together with kidney, heart, brain, and spleen, were stored at -20 degrees until analysis. The metabolism cages were rinsed with 10 ml distilled water, rinses were combined with the urine, and urine was stored under toluene at 4 degrees.

Microsome Preparation. Microsomes were prepared at 4 degrees by a modification of the method of May and McCay (57). Livers were removed from starved rats (those on special diets were not starved), minced in 25 ml of 0.15 M potassium phosphate, pH 7.4, and homogenized with a Potter-Elvehjem homogenizer. Nuclei and cellular debris were removed by centrifuging at 300 x g for 12 min. The supernate was centrifuged again, 20,000 x g for 12 min. The resulting supernate was then centrifuged at 100,000 x g for 90 min in a Beckman Model L2 ultracentrifuge. The pellets were resuspended in fresh 0.15 M potassium phosphate buffer, pH 7.4, and the washed microsomes were centrifuged at 100,000 x g for 30 min. These pellets were resuspended in an appropriate amount of phosphate buffer to produce a solution of 16-20 mg/ml. Microsomal functionality was tested by oxygen uptake according to the method of Ernster and Nordenbrand (58). Protein concentration was determined by the

method of Lowry and coworkers (59) with bovine serum albumin as the standard.

Conditions of Incubations. Microsomes (final concentration 2 mg/ml) were incubated for 4 hr at 37 degrees with 25 mM Tris-HCl, pH 7.4, and 30 mM galactose in a total volume of 1.0 ml. The effects of various levels of activators and inhibitors were tested in the standard incubation, keeping a final volume of 1.0 ml.

Difference Spectra. Microsomal incubations were performed as above. Difference spectra were obtained at zero time and at half hour intervals for the 4 hr incubation period using a Cary spectrophotometer between 200 and 600 nm. Samples without galactose were used as blanks and 0.5% Triton X-100 or 0.5% sodium deoxycholate was used to partially solubilize the microsomes.

Galactonate Isolation. Microsomal incubation mixtures were precipitated by adding 200 μ l of 30% TCA and 20 μ g ribonic acid- δ -lactone were added as an internal standard. Samples were centrifuged to remove precipitated protein and the supernate was extracted three times with three volumes of ether to remove TCA. Tissue samples (0.5 g) were thawed and homogenized in 3 ml of 25 mM Tris-HCl, pH 7.4, containing 20 μ g ribonic acid- δ -lactone, with a Tekmar homogenizer. Protein was precipitated with 1 ml of 30% TCA and samples were centrifuged at 20,000 x g for 10 min. The supernatant fractions were ether extracted as above. Urine samples

collected for 24 hours were brought to 100ml with distilled water after removal of toluene. To 1 ml of urine, 200 ul of 30% TCA and 20 ug of ribonic acid- δ -lactone were added and the samples were then treated as described above.

Galactonate Determinations. Dowex 1-bicarbonate columns (1.3 x 4.5 cm) were prepared in 5 ml disposable syringes equipped with 3-way "luer-loc" stopcocks and equilibrated with 5 mM ammonium bicarbonate, pH 9.0. Microsomal and tissue extracts were diluted with five volumes of 5 mM ammonium bicarbonate, pH 9.0, brought to pH 9.0 with dropwise addition of NH_4OH , and applied to the Dowex columns. Columns were washed with 40 ml 5 mM ammonium bicarbonate, pH 9.0. Galactonate was eluted into 50 ml glass stoppered centrifuge tubes with 15 ml of 100 mM ammonium bicarbonate, pH 9.0, and samples were dried on a rotary evaporator. The samples were resuspended in a mixture of 1 ml water, 3 ml ethanol, and a few drops 0.1 M NaOH to assure retention of the sugar anionic forms. Samples were dried, TMS derivatives prepared, and gas chromatography was performed as outlined above. The Dowex columns were reequilibrated by washing with 25 ml 500 mM ammonium bicarbonate, pH 9.0, followed by 40 ml 5 mM ammonium bicarbonate, pH 9.0. Galactonate levels were determined by comparison of their detector response to that of standard ribonate. Without Dowex treatment of samples galactonate could not be detected owing to the excessive amount of galactose. Removal of galactose allowed detection of as little as 10 nmoles of

galactonate. Gas chromatograms obtained by this method were virtually free of contaminating unknown peaks in the galactonate and ribonate areas.

Hydrogen Peroxide Assay. Hydrogen peroxide was analyzed by a modification of the method of Bernt and Bergmeyer (60). Chromagen reagent was prepared by dissolving 5 mg *o*-dianisidine HCl in 1 ml water and adding 0.5 ml of this solution to 50 ml of 120 mM sodium phosphate buffer, pH 7.4, containing 2 mg horseradish peroxidase. This solution was made fresh and kept in the dark at 4 degrees. Standard hydrogen peroxide solution (2.5 mM) was made daily by dilution of reagent peroxide and determination of exact concentration was by spectrophotometry ($\epsilon_{230\text{ nm}} = 81\text{ M}^{-1}$). A standard hydrogen peroxide curve was prepared by adding 1.0 ml of the chromagen solution to varying peroxide samples. After the reaction was complete in 10 min, 50 μ l of 15% SDS or 100 μ l of 5% Triton X-100 were added. The color at 436 nm was stable for several hours. Microsome incubations were performed as described above except the chromagen-buffer solution was substituted for Tris-HCl. After the 4 hr incubation, 50 μ l of 15% SDS or 100 μ l of 5% Triton X-100 were added to solubilize the microsomes. Blanks were run without galactose.

Protein Separation. Microsomes were prepared as above. Microsomes (2.5 ml) were diluted in 10 ml of 0.15 potassium phosphate, pH 7.4, and 3 ml of 10% Triton X-100 were added to effect solubilization. Ammonium sulfate was added until



a final concentration of 40% was achieved. The solution was stirred on ice for 20 min and centrifuged at 20,000 x g for 12 min. The pellet was resuspended in a minimal volume of 0.15 M potassium phosphate, pH 7.4, containing 0.5% Triton X-100, and dialyzed overnight at 4 degrees against 1 l of the same buffer. The dialyzed solution was passed through a Sephadex G-150 column (1.5 x 45 cm) previously equilibrated with 0.15 M potassium phosphate, pH 7.4, containing 0.5% Triton X-100, and eluted at a rate of 12 ml per hour. Fractions from each step above were assayed by monitoring the rate of formation of hydrogen peroxide using the dianisidine method as outlined above, and employing a Gilford 2000 recording spectrophotometer at 436 nm.



RESULTS

Hemoglobin and Blood Studies. When reduced bovine hemoglobin was incubated with galactose or glucose in the presence of hydrogen peroxide, galactonic and gluconic acids were formed respectively. Aldonic acid levels were not quantified. No sugar oxidation was observed by incubating only hydrogen peroxide and the sugars, but reduced iron, such as ferrous sulfate, could take the place of hemoglobin in the reaction. When red blood cells or plasma were substituted for bovine hemoglobin, no sugar oxidation was observed.

In Vivo Studies. Various levels of galactonic acid were found in the tissues of rats maintained on high galactose diets (Table II). Animals maintained on water containing 0.1% phenobarbital showed a greater concentration of galactonic acid in the liver, blood, and urine than those given distilled water. However, phenobarbital treated animals showed less galactonate in the other organs examined. Overall, the liver had the greatest capacity for galactose oxidation. A large part of the oxidized galactose was excreted in the urine. These findings of galactonate in rat urine are reminiscent of those reported by Bergren, et al (1) after giving human subjects a single oral dose



TABLE II

Concentrations of Galactonate Found in Tissues of Rats
Fed High Galactose Diets and Phenobarbital Water^a

TISSUE	Galactose Diet + Water (nmoles/g)	Galactose Diet + 0.1% Phenobarbital (nmoles/g)
URINE	176 ± 29.9 ^b	412 ± 40.0 ^b
LIVER	3350 ± 243	7650 ± 994
BLOOD	45.2 ± 6.83 ^c	83.1 ± 6.32 ^c
INTESTINE	488 ± 48.1	235 ± 37.0
KIDNEY	346 ± 16.5	332 ± 28.9
BRAIN	244 ± 18.8	142 ± 10.8
HEART	416 ± 34.5 ^d	332 ± 53.0
SPLEEN	127 ± 52.5 ^e	154 ± 52.0 ^d

^aValues represent the mean ± S.D. of 4 animals. Animals were maintained on 0.1% phenobarbital for 10 days, high galactose diets for 3 days. Galactonate was analyzed as outlined in the Methods section. Animals fed control diets showed no galactonate.

^b(umoles/day)

^c(nmoles/ml)

^d_{n=3}

^e_{n=2}



of galactose. Urine and tissues from animals not fed galactose contained no detectable galactonate.

Microsome Activities. Microsomes isolated from animals maintained on the various diets were incubated with galactose. As shown in Table III, feeding a high galactose diet for 72 hours did not induce galactose oxidizing activity. Animals drinking 0.1% phenobarbital and eating control diet showed a decreased specific activity, and those maintained on phenobarbital water and galactose diet showed only 40% of the control activity. The effects of phenobarbital are most likely due to the stimulation of the synthesis of other microsomal proteins especially related to metabolism of the drug. Decrease in specific activities after phenobarbital treatment have been seen for other microsomal proteins such as glucose-6-phosphatase (61-63). The difference in galactose oxidizing activity seen in microsomes isolated from livers of animals drinking 0.1% phenobarbital and eating control diet, and those drinking 0.1% phenobarbital and galactose diet may be attributable to the fact that animals fed the galactose diet drank far more water than those on the control diet. Consequently, animals on the galactose diet ingested more phenobarbital than the controls in the 72 hours before sacrifice.

Cellular Distribution. Galactose oxidizing activity was tested in the mitochondrial and soluble fractions, as well as in the microsomal fraction of rat liver homogenates.



TABLE III

Galactose Oxidation Activity in Rat Liver Microsomes^a

Liver Microsomes	Galactose Oxidation (nmoles/4hr/mg protein)
Control Diet	108 $\pm$ 6.5
Control Diet + 0.1% Phenobarbital	70.9 $\pm$ 16.0
Galactose Diet	101 $\pm$ 11.6
Galactose Diet + 0.1% Phenobarbital	41.3 $\pm$ 6.6

^aValues are mean $\pm$ S.D. for 4 samples. Incubations contained microsomes, 2 mg/ml; galactose, 30 mM; in Tris-HCl, pH 7.4, 25 mM. Galactonate was measured as outlined in the Methods section.



Mitochondrial specific activity was 49% that of microsomal activity. Galactose was not oxidized by the 100,000 x g supernatant fraction.

Galactonate Production with Time, Protein, and Substrate.

Galactonate production was linear with time from 30 to 240 min (Figure 1). Activity in this system was linear with protein concentration for up to 3.1 mg/assay. No activity was observed in boiled (10 min) microsomes. The apparent K_m for galactose from Lineweaver-Burk plots (64) was 32.9 mM and the V_{max} was 160 nmoles of galactonate/4 hr/ mg microsomal protein (Figure 2).

Effect of pH. The galactose oxidizing activity of microsomes was tested over the pH range 3 to 10 using sodium acetate, potassium phosphate, Tris-HCl, and glycine buffers at 25 mM concentrations. No activity was found below pH 5.0 and optimum activity occurred at pH 8.0 (Figure 3).

Activators and Inhibitors. p-Chloromercuribenzoate, iodoacetamide, and N-ethylmaleimide strongly inhibited the microsomal galactose oxidizing activity (Table IV), which suggests that sulfhydryl groups are necessary for activity. However, the sulfhydryl compound dithiothreitol did not enhance the activity, and cysteine was shown to inhibit it. The reaction was completely inhibited by divalent copper; zinc and iron (II) showed some inhibition, but magnesium showed a slight increase in activity. No inhibition was seen with EDTA, and cyanide caused an increase in

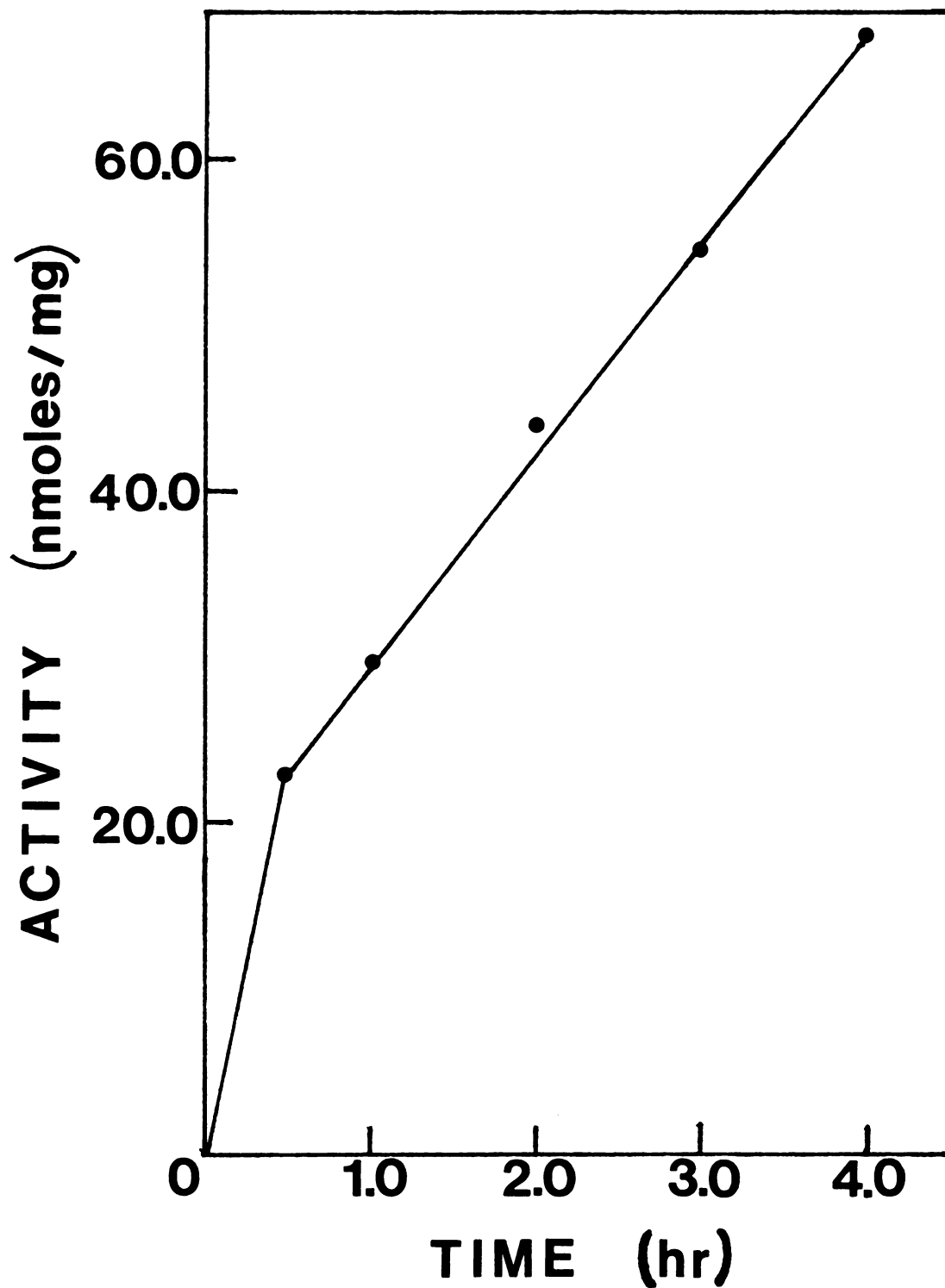


Figure 1: Time course of microsomal galactonate production. The reaction was stopped at the designated times by addition of 200 μ l of 30% TCA. Galactonate was determined as outlined in the Methods section. Each point represents the average of duplicate samples.

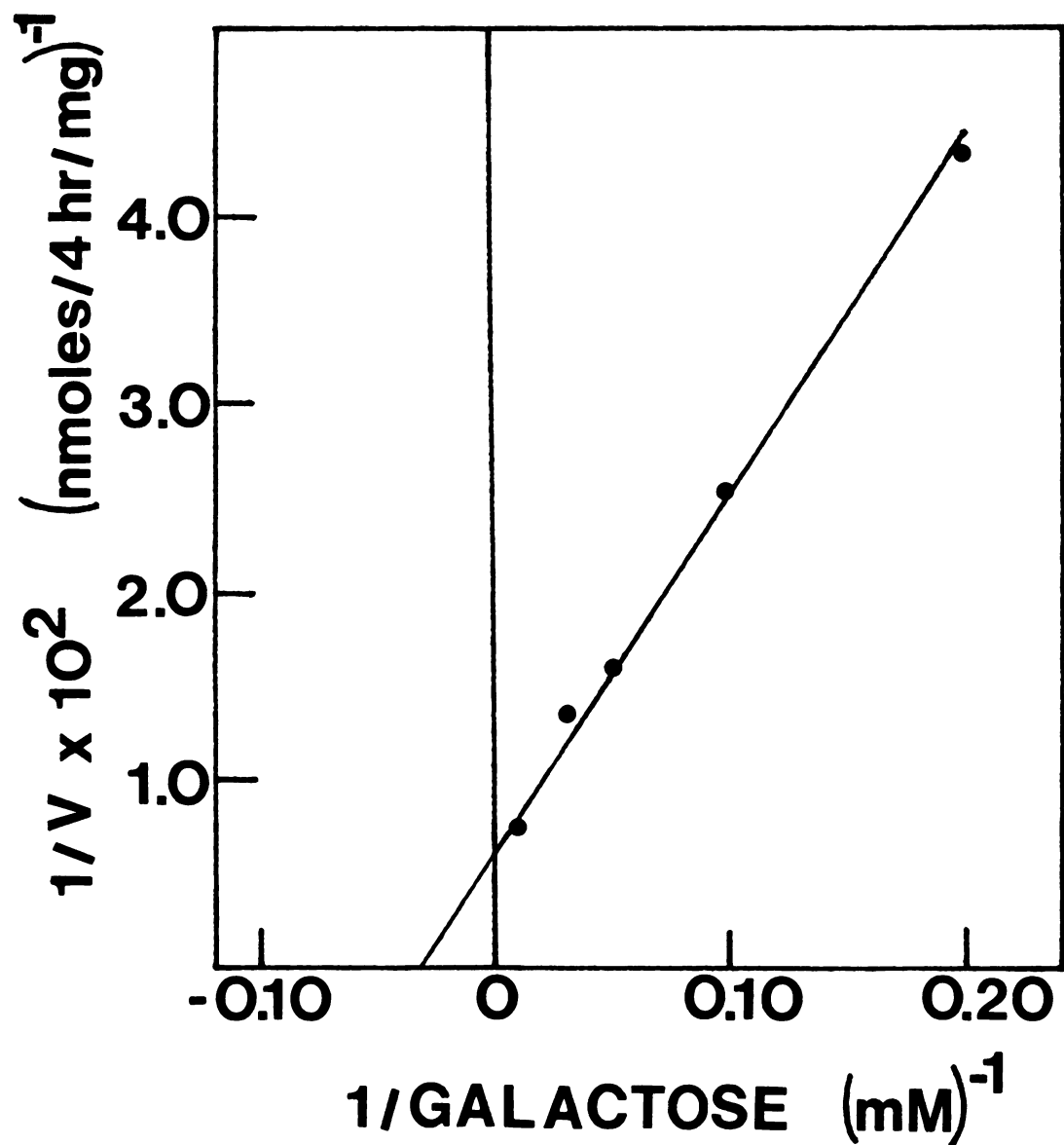


Figure 2: Lineweaver-Burk analysis of kinetic data. Galactonate determinations were performed as described in the Methods section. Each point represents the average of triplicate samples. $K_m = 32.9 \text{ mM galactose}$, $V_{\max} = 160 \text{ nmoles galactonate/4 hr/mg}$.



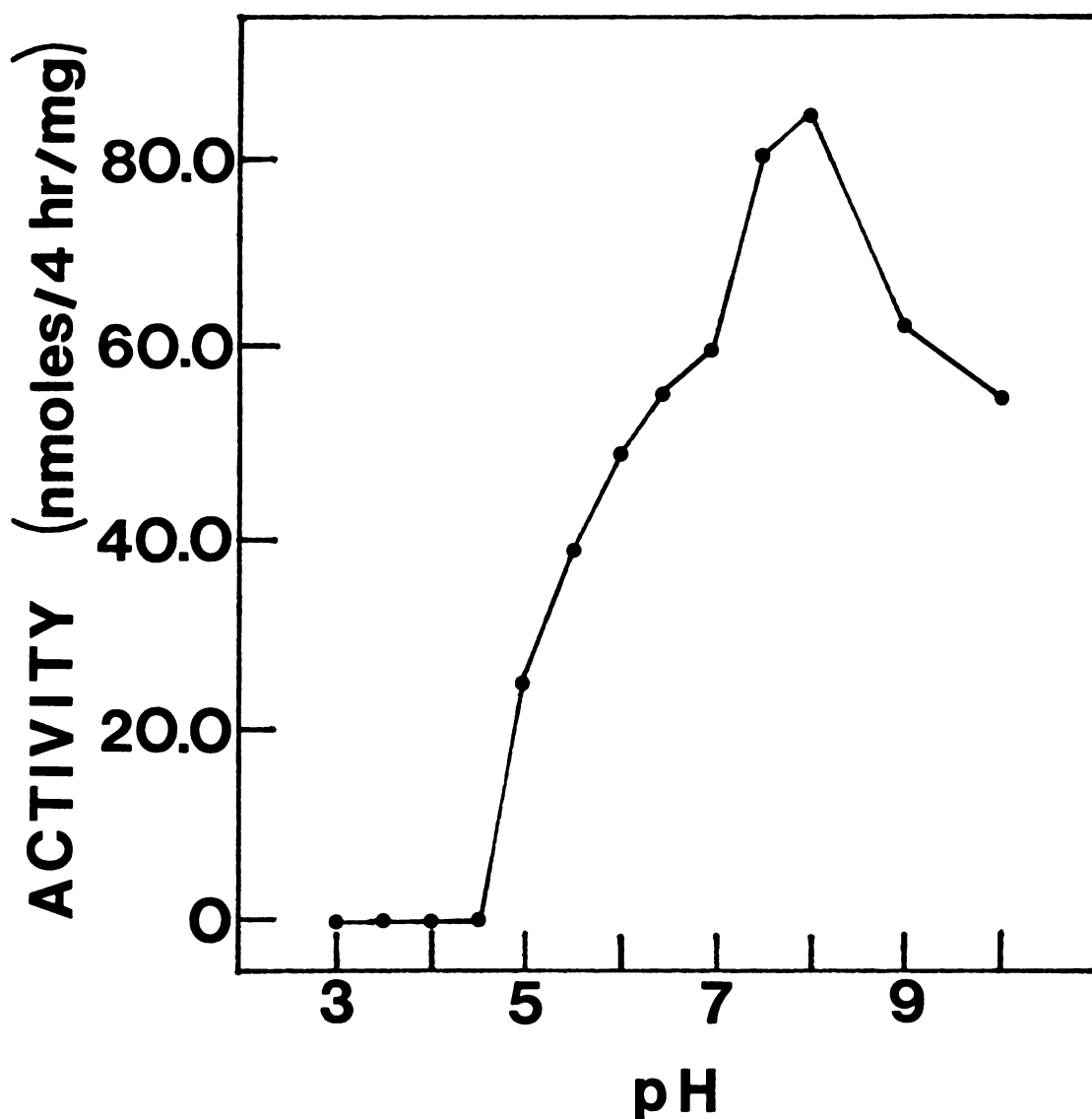


Figure 3: Effect of pH on microsomal galactose oxidation. Acetate buffer was used for pH 3.0-5.5, phosphate buffer for pH 6.0-7.0, Tris-HCl for pH 7.5-9.0, and glycine buffer for pH 10. Galactonate was determined as outlined in the Methods section.



TABLE IV

Effects of Various Activators and Inhibitors on the Galactose Oxidizing Activity of Rat Liver Microsomes^a

Compound	Concentration	Percent of Control	Compound	Concentration	Percent of Control
p-Chloromercuribenzoate	0.5 mM	11.2	Ethanol	30 mM	101.8
Iodoacetamide	1.0 mM	42.3	Mannitol	100 mM	100.0
N-Ethylmaleimide	0.1 mM	30.8	Benzoate	10 mM	57.8
Cystine	1.0 mM	38.0	Thiourea	100 mM	11.9
Cysteine	1.0 mM	55.3	Diphenylfuran	1.0 mM	100.0
Dithiothreitol	0.5 mM	96.9	Diphenylisobenzofuran	1.0 mM	100.0
CuSO ₄	0.5 mM	0	ADP-Fe ³⁺ , NADPH ^b		121.5
ZnCl ₂	0.5 mM	34.4	ADP-Fe ²⁺ , NADPH ^b		42.3
FeCl ₂	0.3 mM	77.0	Pyrazole	10 mM	93.9
MgCl ₂	5.0 mM	106.0	Glutathione	5 mM	100.0
EDTA	5.0 mM	100.0	BHT	5 mM	100.0
KCN	0.2 mM	116.3	Catalase	90 U	122.1
Sodium Azide	1.0 mM	76.9	Superoxide Dismutase	70 U	122.7
Acetone	0.27 mM	50.6	NADPH or NADH	0.37 mM	100.0

^aGalactose oxidation activity was assayed as described under the Methods section. Each value represents the average activity of two to three samples, expressed as percent of the control activity.

^bADP: 0.5 mM
 Fe²⁺, Fe³⁺: 0.06 mM
 NADPH: 0.37 mM

activity, suggesting that metal cofactors are not involved in the activity. Organic solvents had a variable effect; 0.27 mM acetone decreased the activity by 50% while up to 30 mM ethanol had no effect on the activity. The addition of NADPH and NADH had no effect on the oxidation of galactose. An isocitrate/isocitrate dehydrogenase system was used to regenerate NADPH, and NADH was added after every half hour of the incubation.

The effects of several other inhibitors on the microsomal galactose oxidizing system were studied in order to determine if the observed activity was the result of various known oxidative mechanisms. Singlet oxygen trappers, diphenylfuran and diphenylisobenzofuran, had no effect on the galactose oxidizing system. Hydroxyl radical scavengers, benzoate (10 mM) and thiourea (100 mM), showed a 45% and 88% decrease in activity, but mannitol (100 mM) and ethanol (30 mM) had no effect. Thus, these data are not consistent with the involvement of a hydroxyl radical mechanism. Pyrazole (10 mM) was used to rule out alcohol dehydrogenase activity. Addition of lipid peroxidation enhancers, ADP-Fe³⁺ and NADPH, showed a 20% increase in activity, but cyanide did not decrease the oxidative activity, arguing against a lipid peroxidation mechanism. Glutathione and BHT had no effect on the activity, ruling out a spontaneous oxidation. Addition of 90 units of catalase or 70 units of superoxide dismutase caused a 20% increase in activity. Sodium azide (1 mM) decreased activity 25%.



Substrate Specificity. Several sugars were tested as substrates in the microsomal galactose oxidizing system. No oxidation products were found in the gas chromatographic assay with D-glucose, D-mannose, D-allose, D-fructose, D-xylose, D-arabinose, or 2-deoxy-D-glucose as substrates. Unidentified oxidation products were found with D-altrose (15.6% of control), D-talose (17.1% of control) and 2-deoxy-D-galactose (63.8% of control). Incubations with L-galactose showed 13.4% of control activity. The specific rotation of the L-galactose used here (measured with a Zeiss polarimeter after overnight equilibration) was -72.3 degrees, compared with a specific rotation of +83.0 determined with authentic D-galactose. It is possible that much of the activity seen with the L-galactose may be attributable to a D-galactose contaminant.

Difference Spectra. Difference spectra obtained throughout the microsomal incubation period were of little value. The incubation mixture was turbid and the detergents Triton X-100 (0.5%) and sodium deoxycholate (0.5%) were added to solubilize the protein. Samples so treated did not produce useful spectra, and still showed turbidity due to glycogen.

Reaction with Hydrogen Acceptors. Little change in galactonate production was observed with the addition of NAD^+ or NADP^+ to the microsomal assay system (Table V). FAD and FMN (0.37 mM) acted as inhibitors, reducing the galactose oxidizing activity to approximately 50% of the control value.



TABLE V

Effects of Hydrogen Acceptors on the
Galactose Oxidizing System in Rat Liver Microsomes^a

Substance	Concentration	Percent of Control
Oxygen	Ambient	100.0
NAD ⁺	0.37 mM	90.2
NADP ⁺	0.37 mM	92.8
FAD	0.37 mM	50.6
FMN	0.37 mM	48.0
2,6-Dichlorophenolindolphenol	0.20 mM	0

^aGalactose oxidation activity was assayed as described under the Methods section. Each value represents the average activity of two to four samples, expressed as percent of the control activity.



2,6-Dichlorophenolindolphenol (0.20 mM) completely destroyed all galactose oxidation. Taken together, these data strongly suggest that oxygen is the sole hydrogen acceptor of the microsomal galactose oxidizing system.

Formation of Hydrogen Peroxide. Microsome samples were incubated in sodium phosphate buffer, pH 7.4, containing horseradish peroxidase, o-dianisidine hydrochloride, and galactose. In the presence of hydrogen peroxide and peroxidase, o-dianisidine is oxidized by the peroxidase to an orange colored product which can be measured spectrophotometrically. Microsome incubations without galactose were used as blanks and peroxide concentration was determined from a standard curve. Color formation in standard peroxide samples was not affected by high galactose concentrations. Half of the microsome incubation samples were treated with Dowex resin and analyzed for galactonate by gas chromatography; the other half were analyzed for hydrogen peroxide. The galactose oxidizing activity of the microsomes was inhibited by the chromagen, a result also seen in the assay of fungal galactose oxidases (65).

With the microsome preparations it was possible to demonstrate color formation that was dependent on the presence of galactose, but the galactonate to peroxide ratio varied from five to one to one to one. Bean and coworkers (66) have reported that competition between endogenous substrates and o-dianisidine for the horseradish peroxidase make quantitation by this method unfeasible.

Effect of Age. Neonatal rats (20 g) of mixed sex showed the greatest microsomal galactose oxidizing activity (Table VI). Liver microsomes from young adults (130 g), adults (260 g), and very old males (540 g) all showed approximately 62% of the activity observed with neonatal animals.

Distribution in Species. The oxidation of galactose was also observed in mice and beef liver microsomes (Table VII). No activity was observed in guinea pig or chicken liver microsomes.

Protein Separation. Attempts were made to further separate this galactose oxidizing activity from rat liver microsomes. The hydrogen peroxide assay was used to monitor the peroxide forming activity. A summary of the preliminary purification scheme is shown in Table VIII. There was no observable activity in the whole homogenate from rat liver. Ammonium sulfate fractionation of the solubilized microsomal fraction resulted in an increase in the total activity, and a nine fold purification. However, dialysis overnight at 4 degrees resulted in the loss of 66% of the observed activity. No protein could be eluted from the Sephadex G-150 column after washing with three column volumes of buffer.



TABLE VI
Effects of Age on Galactose Oxidation
in Rat Liver Microsomes^a

Animal	Weight (g)	Galactose Oxidation (nmoles/4 hr/mg protein)
Neonate ^b	20	120 ± 12.2
Young Adult ^c	130	76.0 ± 5.6
Adult ^d	260	65.8 ± 1.5
Old ^c	540	82.1 ± 15.3

^aMicrosomes prepared and galactonate analyzed as outlined under the Methods section. n=2

^bMixed sex

^cMales

^dFemales



TABLE VII

Galactose Oxidation in Various Species^a

Species	Activity (% of Control) ^f
Rat	100
Mouse ^b	85.9
Cow ^c	45.3
Guinea Pig ^d	0
Chicken ^e	0

^aMicrosomes prepared and galactonate analyzed as outlined under Methods section. n=3, except cow (n=1), guinea pigs (n=4).

^bMice were 3 months old, C57BL/6J-+/+ strain.

^cBeef liver obtained at slaughter house.

^dGuinea pigs weighed 400-600 g, 1½-6 months old.

^eChickens were 1 week old.

^fActivity is expressed as percent of activity compared to rat control.



TABLE VIII

Purification of the Hydrogen Peroxide
Producing Activity from Rat Liver Microsomes^a

Fraction	Volume (ml)	Protein (mg/ml)	Total Units (nmoles/min)	Specific ^b Activity (units/mg)
Microsomes	13.0	35.8	28.0	0.0614
Ammonium Sul- fate (0-40%)	3.3	21.3	38.6	0.549
Dialysis	2.1	21.3	12.8	0.286

^aHydrogen peroxide formation monitored by using dianisidine-peroxidase assay described under the Methods section. Typical assay mix: 1.0 ml chromagen-peroxidase buffer, 75 μ l of 420 mM galactose, 50-75 μ l protein fraction, 100 μ l of 5% Triton X-100. Blanks were run without galactose.

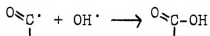
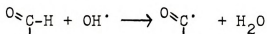
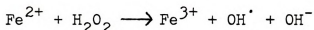
^bUnits defined as nmoles/min.



DISCUSSION

Previous studies of mammalian galactose oxidation have suggested that the activity may be attributed to galactose-6-phosphate dehydrogenase (54), hexose-6-phosphate dehydrogenase (5), or galactose dehydrogenase (2-4).

Galactose-6-phosphate dehydrogenase exhibits high specificity for galactose-6-phosphate and requires NAD^+ for activity. It is found exclusively in liver cytosolic fractions (54). Hexose-6-phosphate dehydrogenase (formerly called glucose dehydrogenase) is a microsomal enzyme and shows minimal activity with galactose. However, this enzyme requires NAD^+ or NADP^+ for activity (5,53). Galactose dehydrogenase required NAD^+ for activity and was a cytoplasmic enzyme (2-4). Its activity was later shown to be due to alcohol dehydrogenase and alcohol contamination of galactose reagents. Cuatrecasas and Segal reported, however, that they were able to isolate radioactive galactonic acid from a system containing liver supernatant fraction, NAD^+ , resorcinol, horseradish peroxidase, galactose-1- ^{14}C , and hydrogen peroxide after 12 hours of incubation (3). Litchfield has shown that galactonate can be easily produced by an in vitro Fenton reaction involving hydroxyl radicals (10):



Galactose incubated in the presence of hydrogen peroxide and ferrous iron was partially converted to galactonic acid. In the reaction system used by Cuatrecasas and Segal, hydrogen peroxide and potential ferrous iron contamination in the reagents or liver supernatant fraction may have generated hydroxyl radicals sufficient to cause the observed oxidation of galactose in vitro.

The results of Litchfield were verified here using the ferrous sulfate incubation system, and it was found that reduced bovine hemoglobin could serve as a source of ferrous iron to generate hydroxyl radicals, partially oxidizing both galactose and glucose to their respective aldonic acids. However, neither intact red blood cells nor plasma could replace the hemoglobin. Apparently the level of catalase found in the red cell is sufficient to destroy the hydrogen peroxide before it can react with hemoglobin.

Galactonate has been identified in the urine of human galactosemics and in the urine of controls given a large galactose load (1). It has not previously been identified to our knowledge in tissues of mammals maintained on high galactose diets. The removal of excess galactose from the tissue extracts through ion exchange chromatography proved important for the successful trimethylsilyl deriva-

tization and detection of the compound. Subsequent gas chromatography increased the sensitivity of the assay over the use of paper chromatography (3,5).

Galactonate was found in the urine and tissues of all rats maintained on high galactose diets. In those animals drinking phenobarbital in addition to the galactose diet, more than two times as much galactonate was found in the liver, blood, and urine. Phenobarbital is known to cause a proliferation of smooth endoplasmic reticulum in liver, followed by an increase in rough endoplasmic reticulum (62,63). This increase in liver microsomal protein may explain the increase in liver galactonate levels found in those animals fed galactose diets and drinking phenobarbital. The higher values seen in the blood and urine of these animals are probably a reflection of the liver values. The action of phenobarbital in other organs is more obscure, but one explanation for the lower galactonate levels observed in all other organs tested in the galactose fed, phenobarbital drinking animals as compared to the animals eating only galactose may be that less galactose is transported to the other organs because of the liver's increased ability in detoxification. This hypothesis could be tested by measuring the galactose levels found in the blood and tissues of the animals eating galactose and drinking 0.1% phenobarbital and those eating only galactose.

When microsomes were isolated from the livers of



the animals fed special diets, it was found that feeding galactose for 72 hours did not induce the galactose oxidizing activity. The addition of phenobarbital to the animals' water resulted in microsomes with decreased specific activities. When 0.1% phenobarbital was added to microsomes prepared from rats eating chow diets, there was no significant effect on galactose oxidizing activity. Phenobarbital induces the synthesis of some microsomal proteins especially related to the metabolism of the drug more than it increases the synthesis of this galactose oxidizing enzyme, resulting in the overall decrease in specific activity.

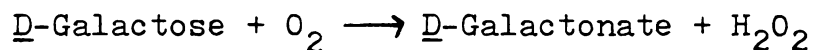
Galactose is oxidized to galactonolactone in a number of other systems. An NAD^+ requiring galactose dehydrogenase found in Pseudomonas saccharophilia (67) and a lactose dehydrogenase from Pseudomonas graveolens (68) are known to show galactonate production. An aerodehydrogenase isolated from citrus fruit oxidizes galactose and several other sugars to the corresponding aldonic acid with simultaneous formation of hydrogen peroxide (66). The mold Polyporus circinatus produces an oxidase that catalyzes the oxidation of galactose at the C-6 position with production of hydrogen peroxide (69,70); this enzyme is now widely used for measuring galactose levels in various tissues.

The microsomal galactose oxidizing activity reported here is not readily attributable to any currently known mechanism. Since singlet oxygen trappers, hydroxyl



radical scavengers, and antioxidants had little effect on galactose oxidation, these diffusion mediated oxidations may be ruled out as sources of activity. Pyrazole, a strong alcohol dehydrogenase inhibitor (71) also had no effect in this system, and the presented data are not consistent with a lipid peroxidation mediated reaction. The high degree of substrate specificity, the use of oxygen as the sole hydrogen acceptor, and the formation of hydrogen peroxide together indicate liver microsomes as a new source of galactose oxidizing activity.

The data here suggest an enzyme that catalyzes a reaction similar to that seen with bacterial glucose oxidase (72-76):



To determine conclusively whether this is the observed reaction, a better method of quantitation of hydrogen peroxide must be sought where endogenous substrates do not compete with the chromagen. A successful separation of the protein from endogenous catalase and peroxidases would also aid in the quantitation of the hydrogen peroxide produced in this reaction. Simultaneous purification of both the galactonate and peroxide producing activities would provide a strong argument for such a reaction.

It is not certain whether galactonate is the initial product of the oxidation, since galactonolactone would be converted to the observed aldonic acid in its anionic form. The increase in activity observed with catalase



and superoxide dismutase provides support for the suggested reaction. Catalase may increase the rate of the forward reaction; azide may inhibit endogenous catalase or the enzyme itself. The increase observed with superoxide dismutase suggests the possibility of a superoxide anion intermediate in the formation of hydrogen peroxide.

Liver microsomes from neonatal rats were found to have the highest galactose oxidizing activities. Older animals showed that the activity decreased with age by nearly 40% and reached a relatively constant value. Since only the neonatal rat is normally exposed to high galactose concentrations in the form of maternal milk, this correlates well with their activity.

It is interesting to note that guinea pig and chicken liver microsomes did not show the galactose oxidizing activity. Chickens are not exposed to dietary galactose, but the enzymes of the uridine nucleotide pathway have been shown to be present in chick liver (77), although the activities are less than those seen in rat liver (78). Guinea pigs would normally encounter galactose in the neonatal diet. There remains the possibility that guinea pig and chicken liver microsomes may exhibit a difference in stability and another method of isolation using sucrose or other membrane stabilizers may be needed.

Further investigation of this galactose oxidizing activity may be directed at finding an assay that is less time consuming than the galactonate assay and more reliable

than the peroxide assay. With a valid peroxide assay, the protein could be further purified. The results reported here are based on microsomal preparations; with a purified protein the effects of various activators and inhibitors may be altered. Another aspect to study would be the correlation of this activity with that seen in a fresh sample of human liver. The presence of this galactose oxidizing activity in human liver would help to explain the excretion of galactonate seen in human galactosemics and in those given a galactose load.

In the dietary studies most of the galactonate appeared to be excreted in the urine, but further metabolism of the compound cannot be ruled out since previous reports have shown the conversion of small amounts of galactose-1-¹⁴C to labelled CO₂ (26,40,79,80). It is also reasonable to suspect that the accumulation of galactonate (ranging from 0.045 to 3.35 mM) may affect the activity of other enzymes in the tissues; Meisler has shown that 1 mM galactonolactone is sufficient to inhibit human liver β -galactosidase by 50% (81) and galactonolactone also competitively inhibits human ganglioside G_{M1} β -galactosidase (82).

Evidence has been presented here for a microsomal enzyme with galactose oxidizing activity. While this activity may be the result of a previously unknown oxidase, the high apparent K_m for galactose suggests that the activity is the result of a microsomal enzyme

with a different primary function. Galactose oxidation may be a reaction called upon when galactose is present in high concentrations, such as in human galactosemia or in galactose tolerance studies.



REFERENCES



REFERENCES

1. Bergren, W. R., Ng, W. G., Donnell, G. N. and Markey, S. P. (1972) Science 176, 683-684.
2. Cuatrecasas, P. and Segal, S. (1966) Science 153, 549-551.
3. Cuatrecasas, P. and Segal, S. (1966) J. Biol. Chem. 241, 5904-5909.
4. Cuatrecasas, P. and Segal, S. (1966) J. Biol. Chem. 241, 5910-5918.
5. Srivastava, S. K. and Beutler, E. (1969) J. Biol. Chem. 244, 6377-6382.
6. Beutler, E. (1967) Science 156, 1516-1517.
7. Shaw, C. R. and Koen, A. L. (1967) Science 156, 1517-1518.
8. Litchfield, W. J. and Wells, W. W. (1976) Infect. Immun. 13, 728-734.
9. Litchfield, W. J. and Wells, W. W. (1977) Infect. Immun. 16, 198-202.
10. Litchfield, W. J. (1976) Ph.D. Thesis, Michigan State University, pp. 121-122.
11. Fong, K. L., McCay, P. B., Poyer, J. L., Keele, R. B. and Misra, H. (1973) J. Biol. Chem. 248, 7792-7797.
12. Penington, J. S. and Prankerdt, T. A. (1958) Clin. Sci. 17, 385-391.
13. Medline, A. and Medline, N. M. (1972) Can. Med. Assoc. J. 107, 877-878.
14. Leloir, L. F. (1951) Arch. Biochem. Biophys. 33, 186-190.
15. Leloir, L. F. (1951) in Phosphorus Metabolism (McElroy, W. D. and Glass, B., eds.), Vol. 1, pp. 67-93, The Johns Hopkins Press, Baltimore.

16. Cardini, L. E. and Leloir, L. F. (1953) Arch. Biochem. Biophys. 45, 55-64.
17. Kalckar, H. M., Braganca, B. and Munch-Petersen, A. (1953) Nature 172, 1038.
18. Atkinson, M., Burton, R. and Morton, R. (1961) Biochem. J. 78, 813-820.
19. Segal, s. (1972) in The Metabolic Basis of Inherited Disease (Stanbury, J. B., Wyngaarden, J. B. and Fredrickson, D. S., eds.), 3rd ed., pp. 174-195, McGraw-Hill Book Company, New York.
20. Mason, H. H. and Turner, M. E. (1935) Amer. J. Dis. Child. 50, 359-374.
21. Hsia, D. Y. Y. and Walker, F. A. (1961) J. Pediat. 59, 872-883.
22. Donnell, G. N., Collado, M. and Koch, R. (1961) J. Pediat. 58, 836-844.
23. Nadler, H. L., Inouye, T. and Hsia, D. Y. Y. (1969) in Galactosemia (Hsia, D. Y. Y., ed.), pp.127-139, Charles C. Thomas, Springfield, IL.
24. Komrower, G. M. and Lee, D. H. (1970) Arch. Dis. Child. 45, 367-373.
25. Donnell, G. N., Koch, R. and Bergren, W. R. (1969) in Galactosemia (Hsia, D. Y. Y., ed.), pp. 247-268, Charles C. Thomas, Springfield, IL.
26. Komrower, G. M., Schwarz, V., Holzel, A. and Golberg, L. (1956) Arch. Dis. Child. 31, 254-264.
27. Holzel, A., Komrower, G. M. and Schwarz, V. (1957) Am. J. Med. 22, 703-711.
28. Holzel, A., Komrower, G. M. and Wilson, V. K. (1952) Brit. Med. J. 1, 194-195.
29. Cusworth, D. C., Dent, C. E. and Flynn, F. V. (1955) Arch. Dis Child. 30, 150-154.
30. Sidbury, J. B. (1960) in Molecular Genetics and Human Disease (Gardner, L. E., ed.), p. 61, Charles C. Thomas, Springfield, IL.
31. Hayman, S. and Kinoshita, J. H. (1965) J. Biol. Chem. 240, 877-882.
32. Moonsammy, G. and Stewart, M. (1967) J. Neurochem. 14, 1187-1193.

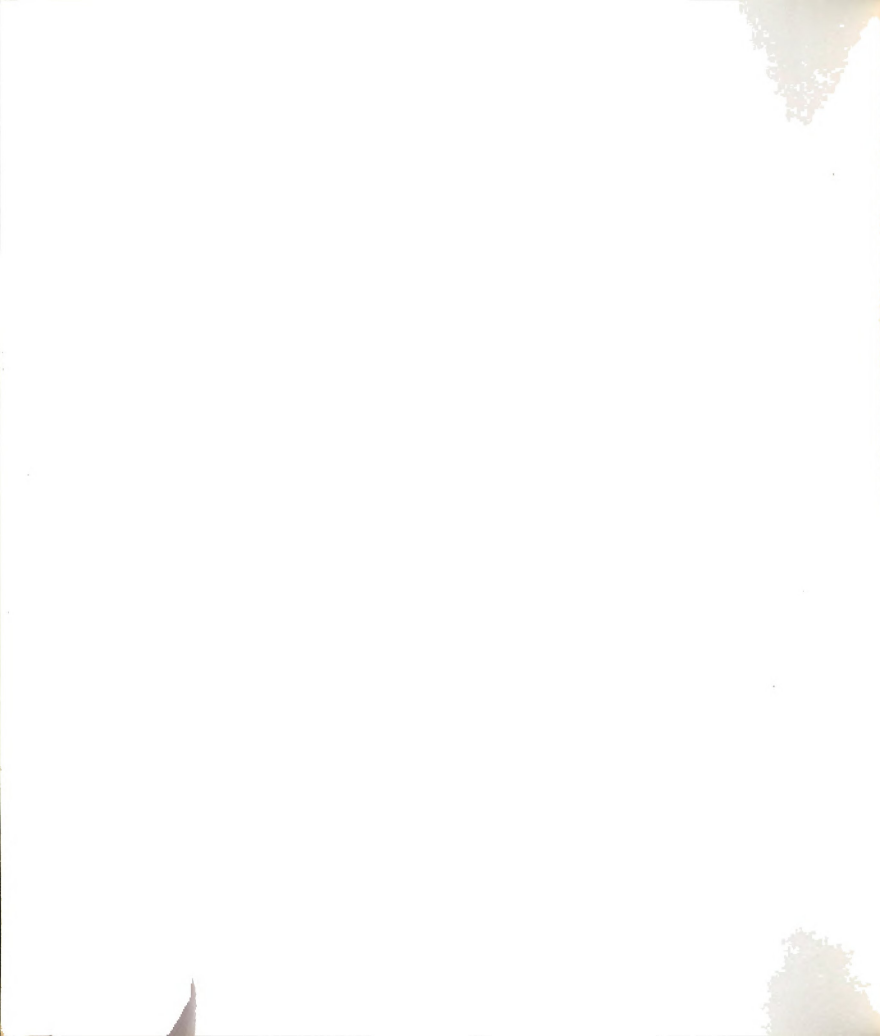


33. Wells, W. W., Pitman, T. A., Wells, H. J. and Egan, T. J. (1965) J. Biol. Chem. 240, 1002-1004.
34. Quan-Ma, R., Wells, H. J., Wells, W. W., Sherman, F. E. and Egan, T. J. (1966) Am. J. Dis. Child. 112, 477-478.
35. Wells, W. W., Pitman, T. A. and Egan, T. J. (1964) J. Biol. Chem. 239, 3192-3195.
36. Weinstein, A. N. and Segal, S. (1968) Biochim. Biophys. Acta 156, 9-16.
37. Kinoshita, J. H. and Merola, L. O. (1964) Invest. Ophthal. 3, 577-584.
38. Sippel, T. O. (1966) Invest. Ophthal. 5, 568-575.
39. Sidbury, J. B. (1969) in Galactosemia (Hsia, D. Y. Y., ed.), pp. 13-29, Charles C. Thomas, Springfield, IL.
40. Segal, S., Blair, A. and Roth, H. (1965) Am. J. Med. 38, 62-70.
41. Segal, S., Blair, A. and Topper, Y. J. (1962) Science 136, 150-151.
42. Petricciani, J. C., Binder, M. K., Merrill, C. R. and Greier, M. R. (1972) Science 175, 1368-1370.
43. Hill, H. Z. and Puck, T. T. (1973) Science, 179, 1136-1140.
44. Isselbacher, K. J. (1957) Science 126, 652-654.
45. Isselbacher, K. J. (1958) J. Biol. Chem. 232, 429-444.
46. Abraham, H. and Howell, R. (1969) J. Biol. Chem. 244, 545-550.
47. Segal, S., Rogers, S. and Holzapple, P. G. (1971) J. Clin. Invest. 50, 500-506.
48. Inouye, T., Tannenbaum, M. and Hsia, D. Y. Y. (1962) Nature 193, 67-68.
49. Ng, W. G. (1964) Ph.D. Thesis, University of Southern California.
50. Posternak, T. and Resselet, J. P. (1954) Helv. Chim. Acta 37, 246-250.
51. Lowry, O. H. and Passonneau, J. V. (1969) J. Biol. Chem. 244, 910-916.

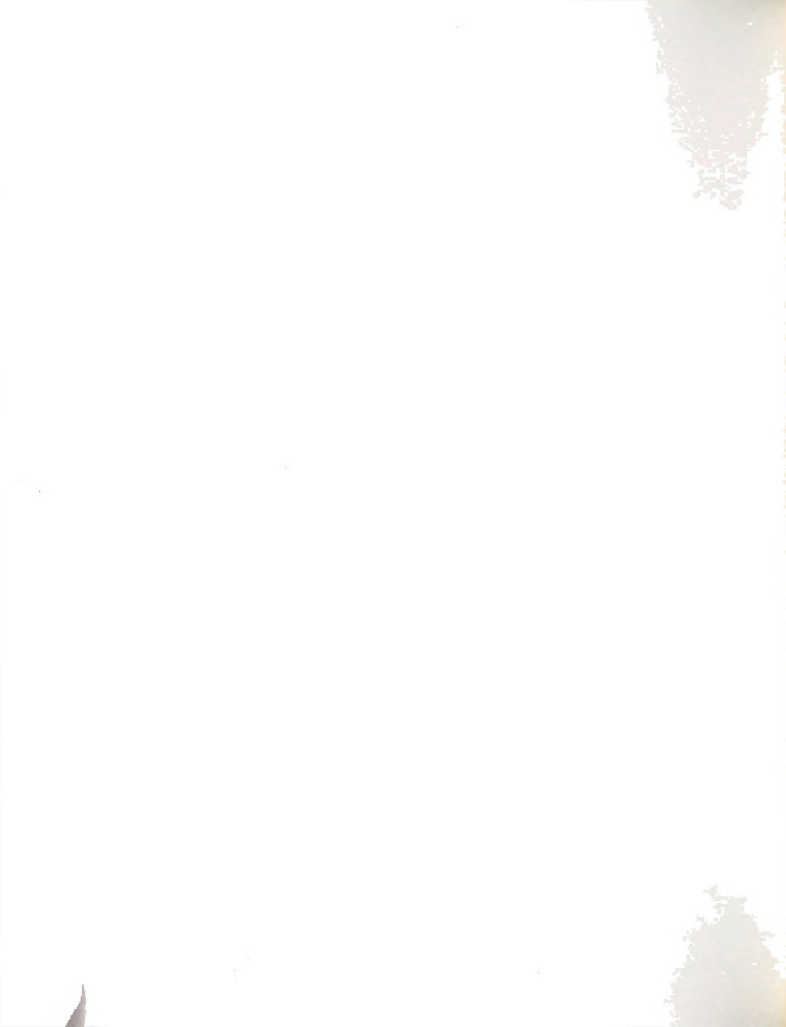
52. Sols, A. and Crane, R. K. (1954) J. Biol. Chem. 210, 581-595.
53. Beutler, E. and Morrison, M. (1967) J. Biol. Chem. 242, 5289-5293.
54. Ray, M. and Bhaduri, A. (1975) J. Biol. Chem. 250, 3595-3601.
55. Sweetley, C. C., Bentley, R., Makita, M. and Wells, W. W. (1963) J. Am. Chem. Soc. 85, 2497-2507.
56. Ross, B. D. (1972) Perfusion Techniques in Biochemistry, p. 26, Clarendon Press, Oxford.
57. May, H. E. and McCay, P. B. (1968) J. Biol. Chem. 243, 2288-2295.
58. Ernster, L. and Nordenbrand, K. (1967) Meth. Enzymol. 10, 574-580.
59. Lowry, O. H., Rosebrough, N. J., Farr, A. D. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
60. Bernt, E. and Bergman, H. U. (1974) in Methods of Enzymatic Analysis (Bergman, H. U., ed.), Vol. 4, pp. 2246-2248, Academic Press, Inc., New York.
61. Stetten, M. R. and Ghosh, S. B. (1971) Biochim. Biophys. Acta 233, 163-175.
62. Orrenius, S. and Ericsson, J. L. E. (1966) J. Cell Biol. 31, 243-256.
63. Orrenius, S., Ericsson, J. L. E. and Ernster, L. (1965) J. Cell Biol. 25, 627-639.
64. Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658-666.
65. Hamilton, G. A., DeJersey, J. and Adolf, P. K. (1973) in Oxidases and Related Redox Systems (King, T. E., et al, eds.), Vol. 1, pp. 103-119, University Park Press, Baltimore.
66. Bean, R. C., Porter, G. G. and Steinberg, B. M. (1961) J. Biol. Chem. 236, 1235-1240.
67. DeLey, J. and Doudoroff, M. (1957) J. Biol. Chem. 227, 745-757.
68. Nishizuka, T. and Hayaishi, I. (1962) J. Biol. Chem. 237, 2721-2728.



69. Cooper, J. A. D., Smith, W., Bacila, M. and Medina, H. (1959) J. Biol. Chem. 234, 445-448.
70. Avigad, G., Amaral, D., Asensio, C. and Horecker, B. L. (1962) J. Biol. Chem. 237, 2736-2743.
71. Teschke, R., Hasumura, Y. and Lieber, C. S. (1976) Arch. Biochem. Biophys. 175, 635-643.
72. Muller, D. (1928) Biochem. Z. 199, 136-170.
73. Muller, D. (1936) Ergebn. Enzymforsch. 5, 259-272.
74. Franke, W. and Lorenz, F. (1937) Liebigs Ann. 532, 1-28.
75. Franke, W. and Deffner, M. (1939) Liebigs Ann. 541, 117-150.
76. Kellin, D. and Hartree, E. F. (1948) Biochem J. 42, 221-229.
77. Mayes, J. S., Miller, L. R. and Myers, F. K. (1970) Biochem. Biophys. Res. Comm. 39, 661-665.
78. Wells, H. J., Gordon, M. and Segal, S. (1970) Biochim. Biophys. Acta 222, 327-332.
79. Topper, Y. J., Laster, L. and Segal, S. (1962) Nature 196, 1006.
80. Baker, L., Mellman, W. J., Tedesco, T. A. and Segal, S. (1966) J. Pediat. 68, 551-558.
81. Meisler, M. (1972) Meth. Enzymol. 28, 820-824.
82. Sloan, H. R. (1972) Meth. Enzymol. 28, 868-874.







MICHIGAN STATE UNIVERSITY LIBRARIES



3 1293 03142 3811